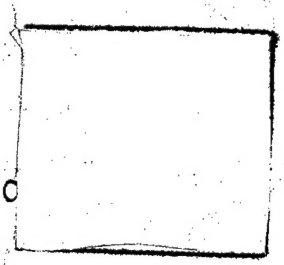

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- USSR -

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MEDIUM FOR DETECTING TOXIC STRAINS OF CL. PERFRINGENS TYPE A, B, C, D, F

[This is a translation of an article written by B. D. Bychenko, Institute of Epidemiology and Microbiology imeni N. F. Gamaley, (Director -- Prof. S. N. Muromtsev), Moscow, in Laboratornoye Delo (Laboratory Affairs), No 1, 1960, pages 40-43]

The simplest methods of isolation of Cl. perfringens (Wilson-Blair media; Robinson-Stoval, high column, etc.) have a substantial defect -- they do not permit the separation of the toxigenic and nontoxigenic strains in the primary inoculation (2). Hence, at the present time, in order to isolate Cl. perfringens from various sources one makes extensive use of the methods based on the utilization of properties of the majority of the toxigenic strains of this microbe to secrete hemolysins and lecithinase (2, 3). The divided inoculation of the investigated material on various indicator media -- dishes with blood-agar, dishes with yolk-agar, etc. (3) -- requires a large consumption of media and laboratory dishware. The specificity of this method is not very high, since many clostridia and other microorganisms are capable of causing hemolysis of erythrocytes and the splitting of lecithin. Divided inoculation of the material does not enable one to state well any certainty that a colony surrounded by zones of hemolysis is capable of a simultaneous alteration of lecithin.

In order to perfect the indicator method of Cl. perfringens isolation, we suggested a combined method based on the principle of growing the suspicious microorganisms between two indicator layers of agar. As indicator plates iron sulfite -- blood -- and yolk-agar were used.

In order to prepare the media, we used: (1) Nutritive meat-peptone-agar (two percent) with glucose (one percent); (2) 10 percent Na_2SO_3 solution (sterilized with flowing stream for one hour); (3) Eight percent $FeCl_3$ prepared on distilled water; (4) Eight percent $PbCH_3COO$ suspension on distilled water; (5) Sterile defibrinated blood of a ram (rabbit or mouse); (6) Chicken eggs. All materials with the exception of blood and eggs can be preserved for a long time (one to two months) in a dark, cool place during the

work. Blood at a temperature of three to six degrees cannot be preserved for more than two weeks, eggs, not over four weeks.

Iron sulfite-blood-agar (ZhSKA). Depending on the projected number of inoculations (calculating 3 ml. of prepared agar to one Petri dish), the nutritive agar was melted in small bottles on a water bath. To 10 ml of hot agar (80-100 degrees) 10 ml of a warm solution of Na_2SO_3 were added and, after the mixture had cooled to 60 degrees, 0.25 ml of $FeCl_3$ solution and 0.5 ml of $PbCH_3COO$ suspension were also added. The mixture was mixed thoroughly and poured into Petri dishes in thin layers (not over one ml); about three to four ml of agar were required to one dish. The dishes were thus used for the inoculation of the material (pure and contaminated Cl. perfringens cultures, exudates and emulsions of tissue from wounds and animal organs which perished of gas gangrene, emulsions of soil, feces, food products, etc.). The material was applied to the plate by means of soft strokes with a platinum loop or with the tip of a Pasteur pipette on a plate of iron-lead-sulfite agar. In three or four places an injection was made to the bottom of the dish. One loop was used to spread the inoculation on two or three dishes. The inoculated dishes were rapidly covered with a thin layer of blood agar (1-2 ml)c which had been prepared as follows. To 100 ml of glucose nutritive agar cooled to 50 degrees were added 10-15 ml of ram's blood, and the mixture was thoroughly stirred.

The agar plates would harden within a few minutes, and there was almost no condensate formed on the dish covers. The inoculations were incubated in a macroanaerostate (37-40 degrees, the atmospheric pressure was up to 600 mm of mercury). The growth of toxigenic Cl. perfringens colonies was observed six to eight hours later, with a characteristic darkening and a hemolysis zone surrounding them. Some Cl. perfringens colonies on the surface of the blood plate rapidly turned green upon contact with air.

Because of the lack of vacuum apparatus under field conditions for the isolation of Cl. perfringens from organs of sheep, this method was employed with a supplementary spread of plain glucose agar (45-50 degrees) in a layer of not less than two mm (25-30 ml on the surface of the blood plate). After the agar had cooled, the dishes were incubated upsidedown in a regular themostat at a temperature of 37-40 degrees. Upon studying the dishes within 10-16 hours under a strong light, the toxigenic Cl. perfringens colonies were seen in the form of smoke-colored or blackened

formations surrounded by a hemolysis aureole.

We also tested the yolk-blood -- and the ironsulfite yolk -- agar. The principle of the method was similar to the one described above, but, in the first instance, we used yolk-agar instead of the iron-sulfite-agar, and, in the second, the inoculated surface, not the blood-agar, was covered with yolk-agar cooled to 45 degrees.

However, from our point of view, iron sulfite-blood agar is the most convenient under laboratory conditions. This medium enables one to isolate Cl. perfringens of any type (by the blackening and hemolysis), while other media (blood-agar, yolk-agar) may not be affected by certain type F strains.

In the subse quent work we used iron sulfite-blood-agar only.

In spite of the fact that there are many microorganisms in existence in nature which are capable of changing iron sulfite-agar (4), the specificity of the method remains sufficiently high, since Cl. perfringens is easily differentiated from the rest of the microflora owing to its rapid growth (six to 12 hours), the presence of a hemolysis zone around the toxigenic colonies, as well as the relatively large sizes of the colonies up to two mm diameters. Besides, in the indicated method of growth the development of obligate anaerobes is inhibited by the incomplete vacuum.

Upon inoculation of materials highly contaminated with some microflora one succeeds occasionally in observing blackened Cl. perfringens colonies in close association with other microbes which do not cause blackening of the medium.

A final identification of the microorganism from a suspicious colony is made after studying the properties of the culture obtained on a liquid medium.

About 300 strains of various types of Cl. perfringens were checked with the foregoing method (A -- 273 strains, B -- six strains, C -- four strains, D -- 16 strains, F -- two strains).

All toxigenic cultures of Cl. perfringens, the filtrates of which killed white mice, caused blackening and hemolysis of the medium following inoculation on iron sulfite-blood agar. Many mildly toxigenic Cl. perfringens cultures, the filtrate of which did not kill white mice, also proved capable of forming blackened colonies with a hemolysis zone.

It is worth noting that Cl. perfringens cultures of types A, B, C, and D, as a rule, changed blood-agar with

the formation of a double hemolysis zone around the colonies (incomplete hemolysis in the outer zone, and complete -- in the inner zone). The type F Cl. perfringens colonies had only an incomplete zone of hemolysis. Non-toxigenic strains of Cl. perfringens did not cause hemolysis, though they did form black or smoke-colored colonies. Ions of iron and lead, in the given concentration, had almost no effect on the toxin formation of Cl. perfringens.

Iron sulfite-blood agar was used in the determination of the presence of toxigenic Cl. perfringens in 120 soil samples from Southern Kazakhstan, where during the spring and fall seasons an infectious enterotoxemia of sheep is observed. The soil was put in sterile test tubes with cotton stoppers and examined after preservation for a period of two to three months (temperature about 20 degrees).

Upon direct inoculation of the soil emulsion in a physiological solution on dishes with iron sulfite-blood-agar the growth of Cl. perfringens was noted only in three out of 45 test tubes. Therefore, all soil samples were inoculated first on a liquid nutritive concentrated medium (five of soil to two flasks with 200 ml of casein medium No 3, TsIEM). One of the flasks was heated to 80 degrees for 15 minutes following inoculation.

After incubation in a thermostat at 37 degrees, the grown, 24-hour cultures were inoculated on dishes with iron-sulfite-blood agar. The Cl. perfringens colonies were isolated within 16 to 24 hours and the properties of fresh six-to seven-hour cultures were then studies. In the absence of specific growth in the flasks (clouding with gas formation), the latter were kept in a thermostat at 27 degrees for several days to grow spores. The grown cultures were examined by the foregoing method.

Thus, according to the symptom of blackening and hemolysis of the iron sulfite-blood agar, we succeeded in isolating Cl. perfringens in 75 percent of soil samples.

The majority of the examined cultures were related to type A and nontoxigenic variants (250 strains), according to the results of activation of the filtrates with trypsin (0.25 percent) and the reaction of neutralization with specific antitoxic sera of A, B, C, D, and F Cl perfringens types. The filtrates of fresh cultures activated with trypsin proved to be highly toxigenic in three strains (from 2,000 to 10,000 Dlm for a white mouse). Before activation with trypsin these filtrates were nontoxigenic or mildly toxigenic and contained no more than 10 Dlm in one lm.

According to the results of the neutralization reaction, all three strains were related to the type D of

Cl. perfringens.

These data may serve as indirect corroboration of the cause of infectious enterotoxemia of sheep in Southern Kazakhstan.

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